B1

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 284 362
A2

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 88302537.1

(22) Date of filing: 23.03.88

(51) Int. Cl.⁴: C 12 Q 1/68
C 07 H 21/00, C 12 P 19/34,
C 07 K 7/10
// G01N33/574, A61K39/395

(30) Priority: 23.03.87 US 29456

(43) Date of publication of application:
28.09.88 Bulletin 88/39

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: IMPERIAL CHEMICAL INDUSTRIES PLC
Imperial Chemical House Millbank
London SW1P 3JF (GB)

(72) Inventor: Elvin, Paul
17 Whitecraigs Place Summerston
Glasgow G23 5LU (GB)

(74) Representative: Mack, John Richard et al
Imperial Chemical Industries PLC Legal Department:
Patents PO Box 6 Bessemer Road
Welwyn Garden City Hertfordshire AL7 1HD (GB)

The microorganism(s) has (have) been deposited with National Collection of Industrial Bacteria under number(s) NCIB 12429

(54) Molecular markers.

(57) The invention relates to polynucleotide sequences and proteins that are differentially expressed during malignant tumour progression and metastasis in colorectal cancer and which may serve as general molecular markers in primary and metastatic neoplastic disease especially colorectal cancer and which may additionally provide a basis for therapy.

EP 0 284 362 A2

5'-AGCGTGGGTA TCGAGGCGGA CGACGACCGG CTCAACAAGG TTATCAGTGA GCTGAATCGA

AAAAACATTC AAGACGTCAT TGCCCAGCGT ATTGGCAAGC TTGCCAGTGT ACCTGCTGGT

GCGGCTGTAC CCGTCTCTGC TGCCCCAGGC TCTGCAGCCC CTGCTGCTG GTTCTGCCCC I

TGCTCCAGCA GAGGAGAAGA CAGATGAGAA GAAGGAGGAG TCTGAAGAGT CAGATGATGA

CATGGGGATT TGCCTTTTTG ATTAAATTCC TGCTCCCCTG CAATAACCTT TTTACACATC

TTA-3' end

It will be appreciated that the fragment of formula I of the present invention may be used to identify mRNA species corresponding thereto in Northern blot analyses.

The polynucleotide sequences of the present invention may also be characterised in that a cDNA sequence derived from said polynucleotide sequences comprises the sequence insert in pLM59 (NCIB No. 12429).

pLM59 has been deposited under the Budapest Treaty with The National Collections of Industrial & Marine Bacteria Ltd, Torry Research Station, PO Box No. 31, 135 Abbey Road, Aberdeen, AB9 8DG, Scotland under the deposition number NCIB 12429. The deposition date in respect of pLM59 is 19th March 1987. pLM59 consists of transformed E.coli JM83 (ATCC NO 35607) See Gene 19, p259-268, 1982 and 25 p241-247, 1983. The insert sequence in pLM59 maybe defined by EcoRI and BamHI restriction sites at terminal ends and by the length of the fragment (see Table 2)

It will be appreciated that the insert sequence in pLM59 or the polynucleotide sequence of formula I hereinbefore defined or a fragment of such sequences having at least 8, preferably at least 10, more preferably at least 12 especially at least 14 consecutive nucleotides may be used to obtain the corresponding genomic DNA or RNA sequences of humans and animals after such sequences have been cloned in appropriate vectors using standard techniques known in the art (see for example T Maniatis et al; Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). Polynucleotide sequences of the present invention may also be prepared by direct chemical or enzymatic synthesis or microbiological reproduction.

It will also be appreciated that polynucleotide sequences of the present invention define the sequences of polypeptides which are encoded therein. The expression of such polypeptides may itself constitute a useful marker in the investigation of malignant disease. Such polypeptides may have some biological role in the development of malignant disease and interference with this function may be useful in therapy of malignant disease.

It will be appreciated that the aforementioned molecular markers may be determined in a number of different ways. Thus for example polynucleotide probes may be constructed which are capable of hybridisation to any portion of the genomic DNA precursor of the aforesaid RNA sequence including introns and non-coding as well as coding portions of the DNA sequence.

Polynucleotide probes may also if desired be constructed which are capable of hybridisation to any portion of the aforesaid RNA sequence, regardless of whether the portion is capable of translation into a polypeptide or not. Moreover, if desired the molecular marker in the form of an RNA sequence may be transcribed into a corresponding cDNA sequence using for example reverse transcriptase and the molecular marker determined by the use of a polynucleotide probe capable of hybridising to any portion of the cDNA sequence. It will be appreciated that the polynucleotide probe will compromise a nucleotide sequence capable of hybridisation to a sufficient length of the sequence to be determined to ensure that the probe unambigously detects the sequence of interest. In general the probe will be capable of hybridising to at least 8 consecutive nucleotides of the sequence to be determined, preferably to at least 10 consecutive nucleotides, more preferably to at least 12 consecutive nucleotides and especially to at least 14 consecutive nucleotides.

Thus according to one feature of the present invention there is provided a polynucleotide probe which comprises a nucleotide sequence capable of hybridising to a polynucleotide of the present invention or portion thereof said probe optionally having a labelled or marker component.

As stated above the polynucleotide probes of the present invention will in general be capable of hybridising to at least 8 consecutive nucleotides of the polynucleotides of the present invention, preferably at least 10 consecutive nucleotides, more preferably to at least 12 consecutive nucleotides and especially to at least 14 consecutive nucleotides.

The polynucleotide probes of the present invention may be labelled or marked according to techniques

known in the art, for example, ^{32}P-radiolabelled in any conventional way, or alternatively radiolabelled by other means well known in the hybridisation art for example to give ^{35}S-radiolabelled probes. The probes may for example carry fluorescent markers. They may alternatively be labelled with biotin or a similar species by the method of D C Ward et al, as described in Proceedings of the 1981 ICN-UCLA Symposium on Development Biology using Purified Genes held in Keystone, Colorado on March 15-20, 1981 vol. XXIII 1981 pages 647-658 Academic Press; Editor Donald D Brown et al or even enzyme labelled by the method of A D B Malcolm et al, Abstracts of the 604th Biochemical Society Meeting, Cambridge, England (meeting of 1 July 1983).

The aforementioned molecular markers may also be determined by the use of antibodies, which may be polyclonal but are preferably monoclonal, raised to a polypeptide sequence coded for by at least a portion of the aforementioned genomic DNA sequence or corresponding RNA sequence. The antibody may thus bind to the protein encoded by the aforementioned genomic DNA sequence or corresponding RNA sequences or bind to any fragment of the protein.

Thus according to a further feature of the present invention there is provided an antibody effective to bind at least a fragment of the polypeptide encoded by the polynucleotide of the present invention. The term "antibody" as used herein includes all immunoglobulins and fragments thereof which contain recognition sites for antigenic determinants of polypeptides of the present invention.

The antibody of the present invention may if desired carry a label or marker component for example as hereinbefore described in relation to the polynucleotide probes of the present invention. Thus the antibodies may for example carry a fluorescent marker. It is not however necessary that the antibody of the present invention carry a label or marker component. Thus for example the antibody of the present invention may be detected by a second antibody which is an antibody to antibodies of the species of the antibodies of the present invention for example goat antimouse immunoglobulin. The second antibody will have a labelled or marker component.

The polynucleotides, polynucleotide probes polypeptides and antibodies of the present invention may find use in the following areas:-

1) Serological diagnosis - for example testing patients, e.g. predisposed to malignancies, for the presence of the protein (encoded by the polynucleotides of the present invention) or antibodies thereto in blood, urine or other body fluids, tissue or excretion products;

2) Immunohistochemistry applications - for the diagnosis of malignant disease in tissue samples

3) Diagnostic imaging - in which case the antibody or probe will have an appropriate label or marker, for example a radioactive label or marker;

4) Therapy - a) for example antibodies of the present invention may form part of an immunotoxin, sometimes termed the "magic bullet", in order to deliver toxic agents of drugs such as plant toxins e.g. ricin preferentially to the site of a malignant or even benign tumour (see for example European Patent Application No. 84304801.8 - Publication No. 0145111);

b) for example the antibodies of the present invention may be useful as a therapeutic;

c) for example polynucleotides of the present invention may be useful alone in therapy as anti sense DNA or RNA. Thus polynucleotides of the present invention, optionally in a vector or in a polynucleotide analogue, which contains sequences complementary to DNA or RNA defining a protein which is differentially expressed during cancer progression and metastasis or portion thereof may be employed to prevent expression of the said protein;

5) Histological analysis - polynucleotide probes (DNA or RNA) having an appropriate label or marker may be useful in in situ hybridisation for histological analysis.

6) Determination of predisposition to genetic disease -for example the polynucleotide of the present invention (DNA or RNA) may be useful in the analysis of restriction enzyme fragment length or other polymorphisms associated with a predisposition to malignant disease. Furthermore, detection of mutations within the polynucleotide sequences of the present invention in individuals may correlate with a predisposition to malignant disease.

Whilst the nucleotide sequences of the present invention were originally identified as being differentially expressed during malignant tumour progression and metastases in colorectal cancer, the nucleotide sequences have been found to be additionally associated with malignant breast disease and metastases. The polynucleotide sequences of the present invention and fragments thereof, the polypeptides of the present invention and fragments thereof and the antibodies of the present invention are thus of interest as general markers in primary and metastatic neoplastic disease and not only in relation to malignant breast disease and colorectal cancer and metastases associated therewith.

Brief description of the drawings

Figure 1 shows Northern blot analyses of normal mucosa RNA. Total RNA (10 µg per lane) from a sample of normal colonic mucosa was electrophoretically fractionated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose. Individual lanes were hybridised with labelled recombinant plasmid probes pNM19, pNM32, pNM41, pNM61 and pLM59 as indicated (19, 32, 41, 61, 59 respectively).

Figure 2 shows Northern blot analyses of RNAs from mucosae, primary colon tumours and liver metastases. Total RNA (10 µg per lane) was electrophoretically fractionated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose and hybridised with ^{32}P-labelled recombinant pLM59 DNA. RNAs were from: lanes 1-4, normal colonic mucosae; lanes 5-7, primary tumours; lanes 8 and 9, liver

metastases; lane 10, normal human liver. RNAs in lanes 1, 2 and 7 were prepared from tissue samples obtained from patients with confirmed metastatic disease. RNAs in lanes 3-6 were prepared from tissue samples obtained from patients with no evidence of secondary disease at the time of surgery.

Figure 3 shows the relative abundance of pNM32 RNA at different stages in colorectal tumour progression. The relative abundance of RNA homologous to recombinant plasmid pNM32 in tissue specimens representing different stages of colorectal tumour progression was determined by doubling-dilution RNA dot-blot hybridisation to ^{32}P-labelled plasmid DNA. Total RNAs at a concentration of 500 µg/ml were diluted and applied to nitrocellulose as described (see Materials and Methods) in a volume of 4 µl, the first dot in each series thus representing 2µg of total RNA.

It may be advantageous to present the polynucleotide probes and/or antibodies of the present invention in the form of diagnostic kits and kits are regarded as further features of the present invention.

Thus according to a further feature of the present invention there is provided a kit for detecting polynucleotides of the present invention which comprises a polynucleotide probe as hereinbefore defined, optionally in labelled or marked form. The kit may additionally contain means for labelling or marking the probes either prior to or subsequent to hybridisation, where such probes are not already labelled or marked. The kit may also contain means for detecting said label or marker. If desired the kit may contain enzymes such as DNA polymerase or enzymes for introducing appropriately labelled nucleotides into DNA or RNA probes. The kit may contain restiction endonucleases and other appropriate materials for performing analyses of RFLP's.

According to a further feature of the present invention there is provided a kit for detecting the polypeptide of the present invention or fragments thereof which comprises an antibody of the present invention as hereinbefore defined, optionally in labelled or marked form. Where the antibody is not in labelled or marked form the kit may contain a second antibody which is an antibody to antibodies of the same species as the unlabelled antibody as hereinbefore described. The second antibody will be labelled or marked and the kit may include a format appropriate for effecting the determination for example as described in US Patent No. 4,376,110. The kit may also contain apparatus for the preparation of histological samples for analysis using antibodies of the present invention.

If desired the polypeptide of the present invention or fragments thereof may be useful as standards in analysis of samples by physical techniques, for example HPLC, TLC or other chromatographic and/or spectroscopic techniques.

According to a futher feature of the present invention there is provided a kit for manufacturing the polynucleotide of the present invention which kit comprises microorganisms containing vectors capable of producing the polynucleotides of the present invention.

For diagnostic imaging the polynucleotide probe or antibody of the present invention will have an appropriate labelled or marker component, for example a radioactive label or marker, and will conveniently be presented in a form suitable for ingestion or injection.

The antibodies of the present invention may also be of interest in purifying a polypeptide of the present invention and accordingly we further provide a method of purifying a polypeptide of the present invention as hereinbefore defined or any portion thereof or a metabolite or degradation product thereof which method comprises the use of an antibody of the present invention.

The purification method of the present invention may be effected by any convenient technique known in the art for example by providing the antibody on a support and contacting the antibody with a solution containing the polypeptide whereby the antibody binds to the polypeptide of the present invention. The polypeptide may be released from binding with the antibody by known methods for example by changing the ionic strength of the solution in contact with the complex of the polypeptide/antibody.

The following non-limiting Example is provided in order to illustrate the present invention:-

EXAMPLE 1

MATERIALS AND METHODS

Tissues

Specimens of histologically confirmed adenomatous polyps, colorectal tumours, and liver metastases from colorectal tumours were obtained from patients undergoing surgery at Glasgow Royal Infirmary. Specimens of histologically normal colonic mucosae were obtained from tissue adjacent to the resection margins of surgically removed colorectal tumours. All tissues were immediately frozen in liquid nitrogen and stored at -70°C until required.

Isolation of total RNA

Total RNA was isolated from the frozen tissue specimens by a modification of the method of Chirgwin et al (1979), which yields undegraded total RNA suitable for the isolation of poly(A)$^+$ RNA, overcoming the high level of activity associated with endogenous RNAases in these tissues. A sample (about 0.51g)of the tissue specimen was ground to a fine powder under liquid nitrogen in a pre-cooled porcelain mortar and pestle. The ground tissue was lysed by transfer to 20 ml of guanidinium thiocyanate solution (5 M guanidinium thiocyanate, 5% mercaptoethanol, 50 mM tris-HCl, 50 mM EDTA, pH 7.0). DNA was fragmented by sonication, 1/10 vol 20% sarcosine added and the solution warmed to 65°C in a water bath for two minutes. Gross tissue debris was

removed by centrifugation at 1000 rev/min for 10 minutes in a MSE 4L centrifuge. The solution was layered over a cushion of CsCl (5.7 M CsCl, 50 mM EDTA, pH 7.0; refractive index 1.3995) and centrifuged at 22,000 rev/min (60 000 g_{av}), at 17°C for 48 hours in an IEC SB-110 rotor.

The pellets were resuspended in sterile water and precipitated by adding 1/10 vol 3 M sodium acetate and 3 vol absolute ethanol. The solution was kept at -20°C overnight, and the precipitated material recovered by centrifugation at 10,000 rev/min (8700 g_{av}) at 4°C for 20 minutes in a Sorvall HB4 rotor. The pellets were washed in 70% and 95% ethanol and finally resuspended in sterile water at a concentration of approx. 1 mg/ml.

Poly(A)$^+$ RNAs were isolated from total RNAs by the method of Aviv and Leder (1972) using oligo(dT)-cellulose (BRL), recovered by precipitation and washed as described above, and finally resuspended in sterile water at a concentration of 250 µg/ml and stored at -20°C.

cDNA library construction

Double-stranded cDNAs were synthesised from poly(A)$^+$ RNAs by the method of Wickens et al (1978). Oligo(dT)-primed poly(A)$^+$ RNA was reverse transcribed by AMV reverse transcriptase (Bio-Rad Laboratories) to generate a first strand with a hairpin loop which was used to prime second strand synthesis by E.coli DNA polymerase I (Boehringer). The hairpin loop was removed by digestion with S1 nuclease and the resultant cDNA was blunt-end ligated into the Sma1 site of plasmid pUC8. The recombinant plasmids were used to transform E.coli JM83 and individual recombinant clones were grown on L-agar 9 cm plates. Individual colonies were picked, inoculated, and grown in 96-well microtitre plates (Flow Laboratories), duplicated and stored at -20°C. Simultaneously, using a transfer plate (Dynatech), two nylon filter (Biodyne A, PALL) replicas of each plate were copied, and the bacterial DNA lysed and baked onto the filters for screening. The insert is recovered by digestion of the recombinant plasmid with Eco RI and Bam HI by methods known in the art.

cDNA probe preparation and colony hybridisation

All of the probes used to screen the libraries were single-stranded cDNAs synthesised from poly(A)$^+$ RNAs using AMV reverse transcriptase (Bio-Rad Laboratories) and ^{32}p-dCTP (α-^{32}p-dCTP, 400 Ci/mmol, Amersham International plc) as label. Colony hybridisation (Grunstein and Hogness, 1975) to the nylon filter replicas of the cDNA libraries was carried out as described by the manufacturer (PALL) at 65°C for at least 12 hours using a probe concentration of 0.5-1x10^6 cpm/ml. Excess probe was removed by three half-hour washes in a washing buffer (5 mM NaH_2PO_4, 1 mM EDTA, 0.2% SDS) at 65°C. Colony hybridisation was visualised by autoradiography at -70°C using Kodak X-Omat film and Dupont Lightening Plus intensifying screens.

Plasmid DNA isolation and dot-blot hybridisation

Small-scale bacterial cultures (2 ml overnight cultures) were used for the isolation of plasmid DNA by the method of Birnboim and Doly (1979). The DNAs were dot blotted onto Biodyne A nylon membrane filters, denatured and baked as described by the manufacturers (PALL) prior to hybridisation under conditions as described for colony hybridisation. For further study plasmids were isolated from 500 ml overnight cultures, using the alkaline lysis method of Birnboim and Doly (1979). The plasmids were purified by CsCl and sucrose gradient centrifugation. Recombinant plasmids were finally resuspended in TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 8.0) at a concentration of 250 µg/ml and stored at 4°C.

Northern blot analysis and dot-blot analysis of total RNA

Total and poly(A)$^+$ RNAs in a buffer solution containing 50% formamide and 2.2 M formaldehyde were heated to 65°C for 10 minutes, chilled on ice, and electrophoretically fractionated on 1% agarose-formaldehyde gels prior to Northern blotting onto nitrocellulose as described by Thomas (1980).

Serial doubling dilutions of total RNAs in sterile water were heated to 65°C for 15 minutes and chilled on ice before dot blotting onto nitrocellulose that had been previously wetted in 20X SSC (2 M NaCl, 0.3 M sodium citrate, pH 7.0) and air dried. The RNAs were immobilised onto the nitrocellulose by baking for 2 hours at 80°C.

Southern blot analysis

Restriction enzyme digested normal human white blood cell DNA, 18 µg per lane, was electrophoretically fractionated overnight on 1% agarose gels, and then transferred to nitrocellulose using a modification of the method of Southern (1975).

Hybridisation conditions

Recombinant plasmids were radioactively labelled by nick-translation using ^{32}p-dCTP (α-^{32}p-dCTP, 400 Ci/mmol, Amersham International plc). Nitrocellulose filters were pre-hybridised in a buffer containing 50% formamide, 0.1% SDS, 5X Denhardt's (0.1% ficol 400K MW, 0.1% polyvinyl pyrolidine 360K MW, 0.1% bovine serum albumin), 5X SSC, 50 mM sodium phosphate , 500 µg/ml salmon sperm DNA, 10 µg/ml each of poly(A) and poly(C), 1% glycine, pH 7.0, for at least 12 hours at 42°C. Hybridisations were carried out in a buffer containing 50% formamide, 10% dextran sulphate, 0.1% SDS, 5X SSC, 1X Denhardts, 20 mM sodium phosphate, 100 µg/ml each of poly(A) and poly(C), pH 7.0, for at least 12 hours at 42°C with a probe concentration of 0.5-1x10^6 cpm/ml. Following hybridisations filters were washed at 65°C in 2X SSC, 0.1% SDS, then 0.5X SSC, 0.1% SDS and finally 0.1X SSC, 0.1% SDS, and exposed to Kodak X-Omat film with

intensifying screens at -70°C.

RESULTS

Screening of cDNA libraries

A cDNA library of approx. 5000 clones representative of normal colonic mucosa poly(A)$^+$ RNAs was screened with probes generated from poly(A)$^-$ RNAs according to the scheme outlined in the screening protocol set out below.

Screening protocol for cDNA libraries: Identification of recombinants associated with tumour stage.

Normal colonic mucosa cDNA library about 5000 clones	Liver metastasis cDNA library about 3000 clones
Colony hybridisation 1	
probes[a]: normal mucosae(4)[b]	probes: liver metastases(2) colorectal ca(4)
912 clones	288 clones
Colony hybridisation 2	
probes: normal mucosae(2) colorectal ca(5)	probes: normal mucosae(3) colorectal ca(3) liver metastases(3) normal liver(1)
89 clones	82 clones
Plasmid DNA dot-blot hybridisation	
probes: normal mucosae(3) colorectal ca(3) liver metastases(3)	probes: normal mucosae(3) colorectal ca(3) liver metastases(3) normal liver(1)

a. All probes were ^{32}P-dCTP-labelled cDNAs reverse transcribed from poly(A)$^+$ RNAs.

Mars, W.M., Florine, D.L., Talpaz, M. and Saunders, G.F. Preferentially expressed genes in chronic myelogenous leukaemia. Blood, 65, 1218-1225 (1985).

Nicolson, G.L. Cancer metastasis: Organ colonisation and the cell surface properties of malignant cells. Biochem. Biophys. Acta, 695, 113-176 (1982).

Nowell, P.C. The clonal evolution of tumour cell populations. Science, 194, 23-28 (1976).

Rothberg, P.G., Spandorfer, J.M., Erisman, M.D., Staroscik, R.N., Sears, H.F., Petersen, R.O. and Astrin, S.M. Evidence that c-myc expression defines two genetically distinct forms of colorectal adenocarcinoma. Br. J. Cancer, 52, 629-632 (1985).

Schirrmacher, V. Shifts in tumour cell phenotypes induced by signals from the microenvironment: Relevance for the immunobiology of cancer metastasis. Immunobiol., 157, 89-98 (1980).

Schirrmacher, V. Cancer metastasis: Experimental approaches, theoretical concepts and impacts for treatment strategies. Adv. Cancer Res., 43, 1-73 (1985).

Schwartz, M.K. Markers in screening: Overview of usefulness of markers. In: Colorectal cancer: Prevention, Epidemiology and Screening. Eds. Winawer, S., Schottenenfeld, D., Sherlock. P. Raven Press, N.Y. (1980).

Shamsuddin, A.K.M. Weiss, L., Phelps, P.C. and Trump, B.F. Colon epithelium: IV. Human colon carcinogenesis: Changes in human colon mucosa adjacent to and remote from carcinomas of the colon. J. Nat. Canc. Inst., 66 , 413-419 (1981).

Shani, M., Nudel, U., Zevin-Sonkin, D., Zakut, R., Givol, D., Katcoff, D., Carmon, Y., Reiter, J., Frischauf, A.M. and Yaffe. D. Skeletal muscle actin mRNA. Characterisation of the 3' untranslated region. Nuc. Acids. Res., 9, 579-589 (1981).

Sim, G.K., Kafatos, F.C., Jones, C.W., Koehler, M.D., Efstratiadis, A., Maniatis, T. Use of a cDNA library for studies on evolution and development expression of the chorion multigene families. Cell, 18, 1303-1316 (1979).

Southern, E.M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., 98, 503-517 (1975).

Spandidos, D.A. and Kerr, I.B. Elevated expression of the human ras oncogene family in premalignant and malignant tumours of the colorectum. Br. J. Cancer, 49, 681-688 (1984).

Stewart, J., Evan, G., Watson, J. and Sikora, K. Detection of the c-myc oncogene product in colonic polyps and carcinomas. Br. J. Cancer, 53, 1-6 (1986).

Suggs, S.V., Wallace, R.B., Hirose, T., Kawashima, E.H. and Itakura, K. Use of synthetic oligonucleotides as hybridisation probes: Isolation of cloned cDNA sequences for human β_2-microglobulin. Proc. Nat. Acad. Sci., 78, 6613-6617 (1981).

Tate, H. Plasma CEA in the post surgical monitoring of colorectal carcinoma. Br. J. Cancer, 46, 323-330 (1982).

Taylor, I., Machin, D., Mullee, M., Trotter, G., Cooke T. and West, C. A randomised controlled trial of adjuvant portal vein cytotoxic perfusion in colorectal cancer. Br. J. Surgery, 72, 355-363 (1985).

Thomas, P.S. Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Nat. Acad. Sci., 77, 5201-5205 (1980).

Thorgeirsson, U.P., Turpeenniemi-Hujanen, T., Williams, J.E. Westin, E.H., Heilman, C.A., Talmadge, J.E. and Liotta, L.A. NIH/3T3 cells transfected with human tumour DNA containing activated ras oncogenes express the metastatic phenotype in nude mice. Mol. Cell. Biol., 5, 259-262 (1985).

Warnock, A.M., Burns, J.H. and Birnie, G.D. Subdivision of the acute non-lymphoblastic leukaemias by measurement of the relative abundance of a specific RNA sequence. Leuk. Res., 9, 955-966 (1985).

Weiss, L. In Principles of metastasis, Acad. Press London 1985.

Welch, J.P. and Donaldson, G.A. Detection and treatment of recurrent cancer of the colon and rectum. Am. J. Surg., 135, 505-510 (1978).

Wickens, M.P., Buell, G.N. and Schimke, R.T. Synthesis of double stranded DNA complementary to lysozyme, ovomucoid, and ovalbumin mRNAs. J. Biol. Chem., 253, 2483-2495 (1978).

Wiedemann, L.M., Burns, J.H. and Birnie, G.D. Differences among the polyadenylated RNA sequences of human leukocyte populations: An approach to the objective classification of human leukaemias. EMBO. J., 12, 9-13 (1983).

Williams, J.G. The preparation and screening of a cDNA clonebank In: Genetic Engineering vol. 1, 1-59 Ed. Williamson, R., Academic Press, London (1981).

Williams, J.G., Hoffman, R. and Penman, S. The extensive homology between mRNA sequences of normal and SV-40 transformed human fibroblasts. Cell, 11, 901-907 (1977).

Wolley, R.C., Schreiber, K., Koss, L.G., Karas, M. and Sherman, A. DNA distribution in human colorectal carcinomas and its relationship to clinical behaviour. J. Nat. Canc. Inst., 69, 15-22 (1982).

Yokota, J., Tsunetsugu-Yokota, Y., Battifora, H., Le Fevre, C. and Cline, M.J. Alterations of myc, myb, and ras^{Ha} proto-oncogenes in cancers are frequent and show clinical correlation. Science, 231, 261-265 (1986).

Table 1. Recombinant cDNA dot blot hybridisation to cDNA probes from histologically graded tissues

Origin of recominant clones	Number of clones[b]	Relative hybridisation to cDNA probes representing[a] Mucosa	Primary tumour	Secondary tumour
Normal colonic	6	+[c]	+	−
mucosa	7	+	+	+/−
cDNA library	7	+	++	+
Liver metastasis	8	+	+	++
cDNA library	6	+	++	+

a. ^{32}P-labelled single-strained cDNAs reverse transcribed from total poly(A)$^+$ RNA.

b. Clones grouped on the basis of hybridisation of plasmid DNA dot blots with probes indicated; identical results obtained with probes derived from three specimins of each tissue type.

c. Hybridisation signals: ++, very strong; +, strong; −, weak or absent.

Table 2. Characteristics of five selected cloned sequences.

cDNA clone	Size of cDNA insert (bp)	Size of homologous RNA (kb)
pNM19	530	1.2
pNM32	485	1.2
pNM41	420	1.9
pNM61	230	2.1
pLM59	400	0.8

Table 3. Relative abundances of five mRNAs in mucosae, polyps, carcinomas and metastases.

Tissues	Recombinant clones				
	pNM19	pNM32	pNM41	pNM61	pLM59
Mucosa (4)[a]	104[b]	320	98	130	5
Polyp (3)	88	192	192	85	4
Carcinoma (4)	107	32	32	32	8
Metastases (2)	10	24	9	9	32

a. Number of individual samples.

b. Mean values of reciprocals of dilution end-points determined by total RNA doubling-dilution dot-blot assay (see fig.4).

Table 4. Abundances of homologous RNAs in metastases relative to mucosa and carcinoma.

Abundance in metastases	Recombinant clone				
	pNM19	pNM32	pNM41	pNM61	pLM59
Relative to mucosa	0.1	0.08	0.09	0.07	6.4
Relative to carcinoma	0.1	0.75	0.28	0.28	4.0

Claims

1. A polynucleotide sequence which is differentially expressed during malignant tumor progression and metastasis in colorectal cancer and fragments thereof.

2. A polynucleotide sequence as claimed in claim 1 characterised in that the cDNA sequence corresponding thereto contains the sequence of formula I as herein defined or a sequence complementary thereto.

3. A polynucleotide sequence as claimed in claim 1 characterised in that a cDNA sequence derived from said polynucleotide sequences comprises the insert sequence in pLM59 (NCIB No. 12429).

4. A process for preparing a polynucleotide sequence as defined in any one of the preceding claims which comprises the use as a probe of the insert sequence in pLM59 or the polynucleotide sequence of formula I as defined in claim 2 or a fragment of such sequences having at least 8 consecutive nucleotides, to obtain the corresponding genomic DNA or RNA polynucleotide sequences as defined in any one of the preceding claims.

5. A process for preparing a cDNA polynucleotide sequence as defined in any one of claims 1 to 3 which comprises synthesising the cDNA sequence from an RNA polynucleotide sequence as defined in any one of claims 1 to 3 by enzymatic techniques known *per se*.

6. A process for preparing a polynucleotide sequence as defined in any one of claims 1 to 3 by chemical or enzymatic synthesis or by microbiological reproduction.

7. A polypeptide or fragment thereof, encoded by a polynucleotide sequence as defined in any one of claims 1 to 3.

8. An antibody effective to bind at least a fragment of the polypeptide as claimed in claim 7.

9. A polynucleotide probe which comprises a nucleotide sequence capable of hybridising to a polynucleotide sequence which is differentially expressed during malignant tumour progression and metastasis in colorectal cancer and fragments thereof.

10. A polynucleotide probe as claimed in claim 9 wherein the nucleotide sequence is capable of hybridising to a polynucleotide sequence as defined in claim 2 or claim 3 or a fragment thereof.

11. A method for the diagnosis or prognosis of malignant disease which comprises detecting the presence or absence in a sample of a polynucleotide or fragment thereof as defined in any one of claims 1 to 3, a polypeptide or fragment thereof as defined in claim 7 or an antibody as defined in claim 8.

12. A method of determining the presence or absence of a predisposition to malignant disease which comprises the use of a polynucleotide or fragment thereof as defined in any one of claims 1 to 3 in detecting the presence or absence of polymorphisms associated with the predisposition to malignant disease.

13. A method of determining the presence or absence of a predisposition to malignant disease which comprises detecting the presence or absence of a mutation within the polynucleotide sequence defined in any one of claims 1 to 3.

Fig.1.

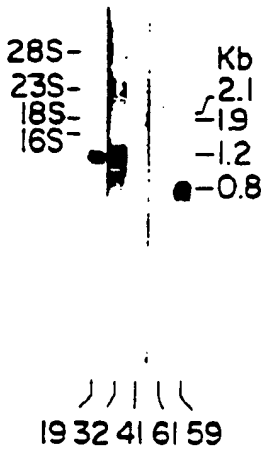


Fig.3.

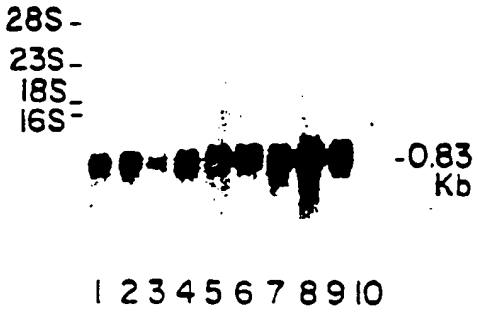
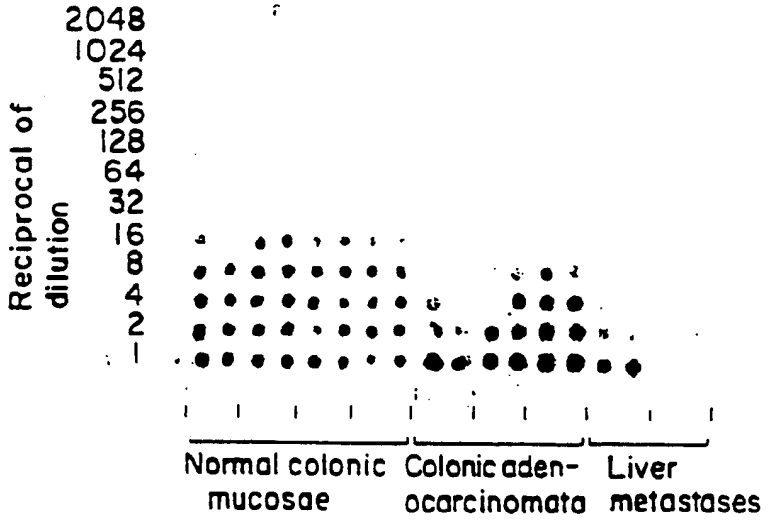


Fig.2.



(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 284 362 A3

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 88302537.1

(22) Date of filing: 23.03.88

(51) Int. Cl.[5] C12Q 1/68 , C07H 21/00 , C12P 19/34 , C07K 7/10 , //G01N33/574,A61K39/395

(30) Priority: 23.03.87 US 29456

(43) Date of publication of application: 28.09.88 Bulletin 88/39

(84) Designated Contracting States: AT BE CH DE ES FR GB GR IT LI LU NL SE

(88) Date of deferred publication of the search report: 04.04.90 Bulletin 90/14

(71) Applicant: IMPERIAL CHEMICAL INDUSTRIES PLC
Imperial Chemical House, Millbank
London SW1P 3JF(GB)

(72) Inventor: Elvin, Paul
17 Whitecraigs Place Summerston
Glasgow G23 5LU(GB)

(74) Representative: Mack, John Richard et al
Imperial Chemical Industries PLC Legal Department: Patents PO Box 6 Bessemer Road
Welwyn Garden City Hertfordshire AL7 1HD(GB)

(54) Molecular markers.

(57) The invention relates to polynucleotide sequences and proteins that are differentially expressed during malignant tumour progression and metastasis in colorectal cancer and which may serve as general molecular markers in primary and metastatic neoplastic disease especially colorectal cancer and which may additionally provide a basis for therapy.

EP 0 284 362 A3

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application number
EP 88302537.1
Page 1

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,X	BRITISH JOURNAL OF SURGERY vol. 70, no. 11, November 1983, GUILDFORD GB page 686;I.B.Kerr et al.: "Isolation of putative metastasis-related cDNA clones from colorectal cancer" * page 686, column 2 *	1,5,9 11	C12Q1/68 C07H21/00 C12P19/34 C07K7/10 //C01N33/574 A01K39/395
D,X	THE BRITISH JOURNAL OF CANCER vol. 49, 1984, LONDON GB pages 681 - 688; D.A.Spandidos et al.: "Elevated expression of the human ras oncogene family in premalignant and malignant tumours of the colorectum" * the whole document"	1,9,11	
D,X	THE BRITISH JOURNAL OF CANCER vol. 52, 1985, LONDON GB pages 629 - 632; P.G.Rothberg et al.: "Evidence that c-myc expression defines two genetically different forms of colorectal adenocarcinoma" * the whole document *	1,9,11	

./..

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C12Q1/00
C12N15/00
C12P19/00
C07H21/00

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely: 1-5,9-13

Claims searched incompletely:

Claims not searched: 6-8

Reason for the limitation of the search: Lack of support in the description

Place of search	Date of completion of the search	Examiner
Berlin	14-12-1989	A.J.DE KOK

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1503.1 03.82

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D,X	CANCER RESEARCH vol. 42, March 1982, CHICAGO US pages 1088 - 1093; L.H.Augenlicht et al.: "Cloning and screening of sequences expressed in a mouse colon tumor" * the whole document"	1,5,9, 11	
A	EP - A - 186522 (SANKYO COMPANY LTD.) * pages 10 - 16; claims 1-11 *	1-5, 9-13	
D,A	THE BRITISH JOURNAL OF CANCER vol. 53, 1986, LONDON GB pages 1 - 6; J.Stewart et al.: "Detection of the c-myc oncogene product in colonic polyps and carcinomas" * the whole document *	1,11, 12	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
T	CHEMICAL ABSTRACTS, vol. 108, no. 17, 25 April 1988 Columbus, Ohio, USA P.Elvin et al.: "Isolation and preliminary characterization of cDNA clones representing mRNAs associated with tumor progression and metastasis" & Br.J.Cancer, vol. 57, no. 1, 1988, pages 36-42; page 200; column 1; ref. no. 1446240 * abstract *	1-5, 9-13	